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(54) Title: HUMAN ALPHA 1,2-MANNOSIDASE

(57) Abstract: The present invention also relates to the use of the recombinant  $\alpha$ 1,2-mannosidase for the development of specific agonist or antagonist of the specific  $\alpha$ 1,2-mannosidase enzyme for specifically converting Man<sub>9</sub>GlcNAc to Man<sub>8</sub>GlcNAc isomer B in degradation mechanism of misfolded proteins, wherein the enzyme has the characteristics of an enzyme encoded by a cDNA sequence set forth in Fig. 1. The present invention also relates to an agonist or antagonist of the  $\alpha$ 1,2-mannosidase enzyme of claim 1 for activating or inhibiting the enzyme. The present invention also relates to a method for the treatment of genetic diseases resulting in misfolding of glycoproteins in a patient, which comprises administering an antagonist of  $\alpha$ 1,2-mannosidase enzyme of claim 1 for transiently inhibiting the enzyme, thereby stabilizing misfolded glycoproteins and preventing their degradation.

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## HUMAN ALPHA 1,2-MANNOSIDASE

**BACKGROUND OF THE INVENTION****(a) Field of the Invention**

5           The invention relates to a novel  $\alpha$ 1,2-mannosidase, and mutated forms, and its therapeutical uses thereof in the treatment of genetic diseases of glycoprotein folding.

**(b) Description of Prior Art**

$\alpha$ 1,2-Mannosidases are essential for hybrid and complex N-  
10   glycan biosynthesis in mammalian cells (Herscovics, A. (1999) Importance of glycosidases in mammalian glycoprotein synthesis. *Biochim. Biophys. Acta*, In press). Following the removal of the glucose residues from the  $\text{Glc}_3\text{Man}_9\text{GlcNAc}_2$  precursor structure attached to nascent glycoproteins, ER and Golgi  $\alpha$ 1,2-mannosidases catalyse the trimming of the four  $\alpha$ 1,2-  
15   linked mannose residues. The subsequent action of GlcNAc transferase I initiates complex chain formation and yields the substrate for Golgi  $\alpha$ -mannosidase II which trims the terminal  $\alpha$ 1,3- and  $\alpha$ 1,6-mannose residues. In some tissues a distinct  $\alpha$ -mannosidase trims  $\text{Man}_5\text{GlcNAc}_2$  to  $\text{Man}_3\text{GlcNAc}_2$  prior to the action of GlcNAc transferase I. Thereafter the N-  
20   glycan structure is further elaborated by Golgi glycosyltransferases.

$\alpha$ -Mannosidases have been classified into two groups based on amino acid sequence homology and on biochemical properties. Class I  $\alpha$ -mannosidases specifically hydrolyze  $\alpha$ 1,2-linked mannose residues, and do not cleave substrates such as p-nitrophenyl- $\alpha$ -D-mannopyranoside.  
25   They require calcium for activity and are inhibited by 1-deoxymannojirimycin and kifunensine, but not by swainsonine. In contrast, Class II  $\alpha$ -mannosidases can cleave  $\alpha$ 1,2-,  $\alpha$ 1,3- and  $\alpha$ 1,6-linked mannose residues as well as p-nitrophenyl- $\alpha$ -D-mannopyranoside and are inhibited by swainsonine, but not by 1-deoxymannojirimycin.

Although several mammalian  $\alpha$ 1,2-mannosidases that can remove up to four  $\alpha$ 1,2-mannose residues have been purified and cloned, there is significant biochemical evidence for the existence of highly specific mammalian enzymes in the endoplasmic reticulum in the endoplasmic reticulum that trim  $\text{Man}_9\text{GlcNAc}_2$  to  $\text{Man}_8\text{GlcNAc}_2$  isomer B, the form lacking the middle-arm terminal  $\alpha$ 1,2-mannose, but mammalian enzymes with this specificity have not yet been purified or cloned. A mammalian ER  $\alpha$ 1,2-mannosidase that forms  $\text{Man}_8\text{GlcNAc}_2$  isomer B and is not sensitive to 1-deoxymannojirimycin was described in intact UT-1 cells and in rat hepatocytes whereas distinct 1-deoxymannojirimycin-sensitive  $\alpha$ 1,2-mannosidase activity that processes  $\text{Man}_9\text{GlcNAc}_2$  to  $\text{Man}_8\text{GlcNAc}_2$  isomer B was observed in the ER of intact COS cells and in ER and in Golgi rat liver membrane preparations. Up to now the yeast ER processing  $\alpha$ 1,2-mannosidase is the only enzyme purified (Jelinek-Kelly, S. and Herscovics, A. (1988) *J. Biol. Chem.*, **263**, 14757-14763) and cloned (Camirand, A. et al. (1991) *J. Biol. Chem.*, **266**, 15120-15127) that specifically trims  $\text{Man}_9\text{GlcNAc}_2$  to  $\text{Man}_8\text{GlcNAc}_2$  isomer B in the endoplasmic reticulum. This enzyme activity triggers degradation of misfolded glycoproteins (Ellgaard, L. et al. (1999) *Science* **286**: 1882-1888).

It would be highly desirable to be provided with the isolation, expression, and properties of a novel human cDNA encoding a Class I  $\alpha$ 1,2-mannosidase that specifically converts  $\text{Man}_9\text{GlcNAc}$  to  $\text{Man}_8\text{GlcNAc}$  isomer B in the endoplasmic reticulum. This enzyme activity triggers degradation of misfolded glycoproteins (Ellgaard, L. et al. (1999) *Science* **286**: 1882-1888).

### **SUMMARY OF THE INVENTION**

One aim of the present invention is to provide the isolation, expression, and properties of a novel human cDNA encoding a Class I  $\alpha$ 1,2-mannosidase of the endoplasmic reticulum that specifically converts  $\text{Man}_9\text{GlcNAc}$  to  $\text{Man}_8\text{GlcNAc}$  isomer B.

In accordance with the present invention there is provided a human  $\alpha$ 1,2-mannosidase enzyme for specifically converting  $\text{Man}_9\text{GlcNAc}$  to  $\text{Man}_8\text{GlcNAc}$  isomer B in degradation mechanism of misfolded proteins, wherein the enzyme has the characteristics of an enzyme encoded by a cDNA sequence set forth in Fig. 1.

In accordance with the present invention there is also provided the tools to develop specific agonist or antagonist of this particular  $\alpha$ 1,2-mannosidase that would not affect the other mannosidases.

The agonist or antagonist may provide for activating or inhibiting for a transient period of time. For example, such an antagonist may be inhibiting the enzyme for a transient period of time, meanwhile preventing misfolded glycoproteins from being degraded, and promoting transport of such glycoproteins from the endoplasmic reticulum to their normal site for function (Marcus, N.Y. et al. (2000) *J. Biol. Chem.* **275**, 1987-1992). Also, changing the specificity of the ER  $\alpha$ 1,2-mannosidase so that it can remove more mannose residues from  $\text{Man}_9\text{GlcNAc}_2$  in the endoplasmic reticulum will also prevent degradation of misfolded glycoproteins. The R461L mutant removes at least three mannose from  $\text{Man}_9\text{GlcNAc}_2$ .

In accordance with the present invention there is also provided the potential for a method for the treatment of genetic diseases causing misfolding of proteins in a patient, which comprises administering an antagonist of  $\alpha$ 1,2-mannosidase enzyme for transiently inhibiting the enzyme, thereby prevent misfolded glycoproteins from degradation.

For example, such a genetic diseases of protein misfolding include, without limitation, cystic fibrosis, emphysema, among others.

The misfolded protein for cystic fibrosis is, for example, cystic fibrosis transmembrane conductance regulator (CFTR) (Ward C.L. et al. (1995) *Cell*, **83**:121-127).

The misfolded protein for emphysema is, for example, alpha1 antitrypsin (Marcus, N.Y. et al. (2000) *J. Biol. Chem.* **275**, 1987-1992).

In accordance with the present invention there is also provided the use of a mutant  $\alpha$ 1,2-mannosidase to produce altered recombinant glycoproteins with improved uptake. Such an improved uptake may be

used to treat genetic diseases characterized by formation of misfolded glycoproteins resulting in their degradation and/or improper localization.

For the purpose of the present invention the following abbreviations are defined below.

- 5 ER endoplasmic reticulum;
- RT reverse transcriptase;
- ORF open reading frame;
- RACE rapid amplification of cDNA ends;
- GSP gene specific primer;
- 10 YPD yeast peptone dextrose;
- BMGY buffered glycerol-complex;
- BMMY buffered methanol complex;
- HPLC high performance liquid chromatography.

#### 15 **BRIEF DESCRIPTION OF THE DRAWINGS**

Fig. 1 illustrates the nucleotide deduced amino acid sequence of the human  $\alpha$ 1,2-mannosidase cDNA;

Fig. 2 illustrates Northern blot analysis of human  $\alpha$ 1,2-mannosidase expression;

20 Fig. 3 illustrates the expression of the recombinant  $\alpha$ 1,2-mannosidase in *P. pastoris*;

Fig. 4 illustrates the time course human  $\alpha$ 1,2-mannosidase activity;

25 Fig. 5 illustrates the [ $^1\text{H}$ ]-NMR spectrum identifying the  $\text{Man}_8\text{GlcNAc}$  isomer produced by the human  $\alpha$ 1,2-mannosidase;

Fig. 6 illustrates the time-dependent removal of at least 3 mannose residues from  $\text{Man}_9\text{GlcNAc}_2$  by the human  $\alpha$ 1,2-mannosidase R461L mutant; and

30 Fig. 7 illustrates the order of removal of mannose from  $\text{Man}_9\text{GlcNAc}_2$  by the human  $\alpha$ 1,2-mannosidase R461L mutant.

## DETAILED DESCRIPTION OF THE INVENTION

In accordance with the present invention, there is provided the isolation of a novel human cDNA encoding a type II membrane protein of 79.5 kDa with amino acid sequence similarity to Class I  $\alpha$ 1,2-mannosidases. The catalytic domain of the enzyme was expressed as a secreted protein in *Pichia pastoris*. The recombinant enzyme removes a single mannose residue from  $\text{Man}_9\text{GlcNAc}$  and [ $^1\text{H}$ ]-NMR analysis indicates that the only product is  $\text{Man}_8\text{GlcNAc}$  isomer B, the form lacking the middle-arm terminal  $\alpha$ 1,2-mannose. Calcium is required for enzyme activity and both 1-deoxymannojirimycin and kifunensine inhibit the human  $\alpha$ 1,2-mannosidase. The properties and specificity of this human  $\alpha$ 1,2-mannosidase are identical to the endoplasmic reticulum  $\alpha$ 1,2-mannosidase from *Saccharomyces cerevisiae* and differ from those of previously cloned mammalian (mouse and human) Golgi  $\alpha$ 1,2-mannosidases that remove up to four mannose residues from  $\text{Man}_9\text{GlcNAc}_2$  during *N*-glycan maturation. Northern blot analysis showed that all human tissues examined express variable amounts of a 3 kb transcript. This highly specific  $\alpha$ 1,2-mannosidase is likely to be involved in glycoprotein quality control since there is increasing evidence that trimming of  $\text{Man}_9\text{GlcNAc}_2$  to  $\text{Man}_8\text{GlcNAc}_2$  isomer B in yeast and mammalian cells is important to target misfolded glycoproteins for degradation. Furthermore, it has been shown that inhibition of this enzyme activity prevents the degradation and increases secretion of improperly folded mutant alpha1-antitrypsin characteristic of emphysema.

### **Materials**

Oligonucleotides were synthesized by BioCorp (Montréal, Canada).  $\text{Man}_9\text{GlcNAc}$  was prepared from soya bean agglutinin and [ $^3\text{H}$ ]mannose-labeled  $\text{Man}_9\text{GlcNAc}$  from rat liver as described previously (Jelinek-Kelly, S. and Herscovics, A. (1988) *J. Biol. Chem.*, **263**, 14757-14763).  $\text{Man}_8\text{GlcNAc}$  isomer B substrate was prepared by digestion of

Man<sub>9</sub>GlcNAc with the yeast  $\alpha$ 1,2-mannosidase. 1-deoxymannojirimycin, kifunensine and swainsonine were obtained from Toronto Research Chemicals, Inc. (Downsview, Canada). All other chemicals were reagent grade.

5

#### **Isolation of a novel human $\alpha$ 1,2-mannosidase cDNA**

The NCBI dbEST database was searched with the yeast  $\alpha$ 1,2-mannosidase amino acid sequence (Camirand, A. et al. (1991) *J. Biol. Chem.*, **266**, 15120-15127) using the tBLASTn algorithm to identify novel  
10  $\alpha$ 1,2-mannosidases. Retrieved ESTs were aligned using the DNASTAR SeqMan program (Madison, WI) and EST clones AA631254, R16652 and H46222 encoding the 3' region of the ORF were obtained from Genome Systems Inc. and sequenced. The following nested primers were designed based on the consensus sequence, 5'-CCACAGACCCAGCAAGGTGCC-3'  
15 and 5'-CTAGGCAGGGGTCCAGATAGG-3', and used to amplify clones containing additional 5' sequence from human placenta Marathon Ready cDNA (CLONTECH) according to the recommended protocol. A 5  $\mu$ l aliquot the PCR reactions was subcloned into pCRII using the original TA cloning kit (Invitrogen) and clones encoding  $\alpha$ 1,2-mannosidase were identified by  
20 hybridization of colony lifts with gene specific <sup>32</sup>P-labeled oligonucleotide probes. The longest clones (2.1 kb) were sequenced.

Thereafter the Gibco 5' RACE System (Version 2) was used to isolate additional 5' sequence. First strand cDNA was synthesized from 2.5  $\mu$ g of placenta, testis, and liver total RNA (CLONTECH) at 55°C using  
25 ThermoScript RT (Gibco) and a gene specific primer (GSP1 5'-GGGCACTTCTGCTCTTCTTGAAG-3') located within the 5' region of the placenta cDNA clones. Nested 5' RACE amplicons were then generated using gene specific primers (GSP2 5'-ATGACTGTCCTCTGCGGATCTC-3', and GSP3.1 5'-TGTCTTCTGTGACGAAATCTC-3' or GSP3.2 5'-  
30 CAGAGCTTTCCAATGGTCAGC-3' or GSP3.3 5'-TCATAGCTCTCGCCA AAGCTCAGC-3') and Platinum Taq (Gibco) according to the



recommended protocol. The amplicons were subcloned into pCR2.1 (Invitrogen), identified by hybridization of colony lifts with gene specific <sup>32</sup>P-labeled oligonucleotide probes, and sequenced. In addition, Genome Systems isolated three fetal brain  $\alpha$ 1,2-mannosidase cDNA clones (2.7 kb) by a PCR and hybridization cDNA library screen using primers located within the 5' ORF (5'-ATCGGGACTTCACCTCGGTG-3' and 5'-CAGAGC TTTCCAATGGTCAGC-3').

### Northern blot analysis

The human  $\alpha$ 1,2-mannosidase EST R16652 was labeled with [ $\alpha$ -<sup>32</sup>P]dCTP (3000 Ci/mmol) using the multiprime DNA labeling kit (Amersham). The probe was hybridized to human multiple tissue Northern blots (CLONTECH) according to the recommended protocol and exposed to x-ray film for 5 days (Kodak).

### Expression of the catalytic domain in *Pichia pastoris*

The DNA sequence encoding the soluble catalytic domain of the  $\alpha$ 1,2-mannosidase was amplified from a cDNA clone by PCR using the sense oligonucleotide 5'-AAAGAATTCCAGATTAGACCCCAAGCCA AG-3' containing an EcoRI site and the antisense primer SHMSTOP 5'-AAATCTAGACTAGGCAGGGGTCCAGATAGG-3' containing the stop codon followed by an XbaI site. Expression vector pZ $\alpha$ ASHM169 was constructed by ligation of the amplicon into the EcoRI/XbaI sites of pPICZ $\alpha$  A (Invitrogen) in frame with the  $\alpha$ -factor secretion signal. Similarly, shorter untagged (pZ $\alpha$ ASHM240) and tagged (pZ $\alpha$ ASHM240T) expression constructs were prepared using sense primer 5'-AAAGAATTCCAGGGGCACACCAGTGCATCTG-3' and antisense primers SHMSTOP, and SHMR 5'-AAATCTAGAGCAGGGGTCCAGATAGGCAG-3' respectively. Primer SHMR lacks a termination codon thus the C-terminal of the recombinant enzyme was fused to the (His)<sub>6</sub> and Myc tags encoded by pPICZ $\alpha$  A.

*Pichia pastoris* strain GS115 (*his4*) (Invitrogen) was transformed by electroporation with 10 µg of the expression constructs linearized with PmeI. Transformants were grown and assayed for α1,2-mannosidase activity as described previously.

5

### α-Mannosidase Assays

To characterize the recombinant α1,2-mannosidase, medium containing the enzyme was concentrated 7.5 fold using centrifugal filters (Millipore) and equilibrated in 40 mM PIPES pH 6.5. Two microlitres of the  
10 concentrated medium was incubated with [<sup>3</sup>H]mannose-labeled Man<sub>9</sub>GlcNAc at 37°C and the amount of released [<sup>3</sup>H]mannose was determined by the Con A/PEG precipitation method.

Divalent cation requirements were studied by including 0.05 mM EDTA in duplicate assays in the absence or presence of 2 mM CaCl<sub>2</sub>,  
15 ZnCl<sub>2</sub>, MnCl<sub>2</sub>, MgCl<sub>2</sub>, and CoCl<sub>2</sub>. The enzyme was incubated for 2 hours with 5000 cpm of [<sup>3</sup>H]Man<sub>9</sub>GlcNAc in 40 mM PIPES pH 6.5, 1 mg/ml BSA, and 1 mM NaN<sub>3</sub>.

The K<sub>m</sub> was determined by Lineweaver-Burk analysis. Duplicate  
20 30 min assays contained Man<sub>9</sub>GlcNAc substrate (0.05-0.5 mM), 5000 cpm of [<sup>3</sup>H]Man<sub>9</sub>GlcNAc, 1 mM CaCl<sub>2</sub>, 40 mM PIPES pH 6.5, 1 mg/ml BSA, and 1 mM NaN<sub>3</sub>.

The effects of the Class I α-mannosidase inhibitors 1-deoxymannojirimycin and kifunensine, and the Class II α-mannosidase inhibitor swainsonine were investigated by preincubating the enzyme on  
25 ice for 30 min with the inhibitor in 40 mM PIPES pH 6.5, 1 mM CaCl<sub>2</sub>, 1 mg/ml BSA, and 1 mM NaN<sub>3</sub>. Substrate (0.8 mM Man<sub>9</sub>GlcNAc and 20,000 cpm of [<sup>3</sup>H]Man<sub>9</sub>GlcNAc) was then added to the duplicate assays and the mixtures were incubated at 37°C for 1 hour.

The time dependent formation of products was analyzed by  
30 assaying the recombinant enzyme at 37°C in a 30 µl mixture containing 3.4 mM Man<sub>9</sub>GlcNAc, 30,000 cpm [<sup>3</sup>H]Man<sub>9</sub>GlcNAc, 44 mM potassium

phosphate pH 6.5, 1 mg/ml BSA, 1 mM NaN<sub>3</sub> and 12 µl of unconcentrated medium. At 0, 1, 2, 4, 8, and 19.5 h one-sixth of the reaction mixture was collected. The products were resolved by HPLC, and identified by comparing their elution to that of the [<sup>14</sup>C]Glc<sub>3</sub>Man<sub>9</sub>GlcNAc internal  
5 standard, as described previously.

### High Resolution [<sup>1</sup>H]-NMR Analysis

Medium containing the human α1,2-mannosidase was incubated at 37°C with 600 µg Man<sub>9</sub>GlcNAc and 10<sup>5</sup> cpm [<sup>3</sup>H]Man<sub>9</sub>GlcNAc in 44 mM  
10 potassium phosphate buffer pH 6.5 containing 1 mg/ml BSA and 1 mM NaN<sub>3</sub>. The incubation was supplemented with additional enzyme after 8 and 22.5 h, and terminated after 28.5 h by boiling for 3 min. An aliquot of the sample was analyzed by HPLC to show that most of the sample was transformed to Man<sub>8</sub>GlcNAc. The sample was then chromatographed on a  
15 Bio-Gel P-6 column (1 X 109 cm) equilibrated in deionized water. Fractions containing the oligosaccharide product were pooled, lyophilized, resuspended in D<sub>2</sub>O and lyophilized four times, and stored over P<sub>2</sub>O<sub>5</sub> in a vacuum desiccator. The [<sup>1</sup>H]-NMR spectra were recorded at Université de Montréal NMR Facility in 5 mm tubes using a 600 MHz Bruker  
20 spectrometer at 30 and 70°C with the acetone chemical shift set to 2.225 ppm with respect to 4,4-dimethyl-4-silapentane sulfonate.

### Polyacrylamide gel electrophoresis Western blotting

SDS-PAGE was performed using the Bio-Rad Mini-Protean II  
25 apparatus as described by Laemmli. Western blots were prepared by transferring proteins onto a nitrocellulose membrane (Schleicher and Schuell), and expression of the Myc-tagged recombinant α1,2-mannosidase was detected using the monoclonal Anti-myc Antibody (Invitrogen) and visualized by the ECL Western blotting detection system  
30 (Amersham).

### DNA Sequencing and Alignments

Manual sequencing was performed using the Pharmacia T7 and Deaza sequencing kits. The Sheldon Biotechnology Centre Automated Sequencing Facility (McGill University, Montréal, Canada) employed the  
5 ABI prism dye terminator and thermo sequenase fluorescent labeled primer cycle sequencing kits and the samples were run on the ABI 373A (Perkin Elmer) and ALFexpress (Amersham Pharmacia Biotech) sequencers respectively. The DNASTAR SeqMan program (Madison, WI) was used to assemble the sequences into contigs. The deduced amino acid sequences  
10 were aligned using the BestFit and Publish programs (Version 9.1) from the University of Wisconsin Genetics Computer Group (Madison, WI).

### Preparation of the human R461L ER -mannosidase mutant for expression in *Pichia pastoris* and in mammalian cells

15 The arginine in position 461 of the human ER  $\alpha$ 1,2-mannosidase cDNA clone was changed to leucine with the QuikChange™ Site-Directed Mutagenesis Kit from Stratagene (La Jolla, CA, USA). The sense and antisense primers used for producing the R461L mutant were:  
20 5'GTATTCACGCTGGGCGCTTTGGCCGACAGCTACTATG3' and 5'CAT-AGTAGCTGTCGGCCAAAGCGCCCAGCGTGAATAC3', respectively. These oligonucleotides include a silent mutation deleting an Ehe I digestion site that was used to screen for positive clones. The methylated, non-mutated parental DNA template was digested with Dpn I and *E. coli* DH5 cells were transformed with the nicked, mutated dsDNA. Screening  
25 for positive clones was achieved by using Ehe I (see above), BamH I and EcoR I digestions of the isolated and purified plasmid DNA. The DNA sequence of the positive clones (R461L mutants) encoding the soluble catalytic domain of the  $\alpha$ 1,2-mannosidase (nucleotides 505-2097 corresponding to amino acids 169-699) was amplified by PCR using the  
30 sense primer, 5'AAAGAATTCCAGATTAGACCCCAAGCCAAG3' containing an EcoR I site, and the antisense primer, 5'AAATCTAGACTAGGCAGGGGTCCAGATAGG3' containing the stop codon followed by an Xba I site. The amplicons were subcloned into pCR2.1 (TA Cloning® Kit, Invitrogen). The expression vector was

constructed by digesting the pCR2.1-subclones with EcoR I/Xba I and ligation of the purified insert into the EcoR I/Xba I sites of pPICZA (Invitrogen) in frame with the -factor secretion signal. A tagged expression construct was prepared by using the antisense primer C5PR  
5 5'AAATCTAGAGCAGGGGTCCAGATAGGCAG3' containing the Xba I site but lacking a termination codon. Thus the C-terminal of the recombinant enzyme was fused to the (His)<sub>6</sub> and Myc tags encoded by pPICZA. These constructs were expressed in *Pichia pastoris*, as described above, to obtain the R461L mutant catalytic domain of the human ER  
10  $\alpha$ 1,2-mannosidase.

Similarly, untagged and tagged full length constructs of the wild type ER  $\alpha$ 1,2-mannosidase and the R461L mutant were prepared for expression in mammalian cells. The DNA sequence encoding the full length enzyme was amplified from the mutated cDNA by PCR using the  
15 sense oligonucleotide, 5'AAAAAAGCTTCCACCATGGCTGCCTGCGAGGGCAGGAG3' containing a Hind III site and the antisense primers 5'AAAAAAAAGCGGCCGCTAGGCAGGGGTCCAGATAGGCAGAG3' (containing a Not I site and a termination codon) and 5'AAAAAAAAGCGGCCGCGAGGCAGGGGTCCAGATAGGCAGAG3' (containing a Not I site, no stop codon), respectively. After subcloning the  
20 amplicons into pCR2.1 (TA Cloning<sup>®</sup> Kit, Invitrogen) the full length inserts were ligated into the Hind III/Not I sites of pMH (Roche). Since the last antisense primer lacks a termination codon, the C-terminal of the recombinant enzyme was fused to the HA tag encoded by pMH. With the  
25 same primers tagged and untagged constructs of the full length parental enzyme were prepared. These expression constructs for the full length enzyme are used to examine the effect of additional mannose removal in the endoplasmic reticulum on the degradation and localization of misfolded glycoproteins.

30

### Specificity of the R461L mutant catalytic domain by HPLC

For identification of oligosaccharides products formed by the R461L mutant catalytic domain expressed in *Pichia pastoris*, 10 $\mu$ L of medium were incubated at 37°C for different times in a total volume of  
35 21.5 $\mu$ L containing 10 pmol PA-Man<sub>9</sub>GlcNAc<sub>2</sub>, 0.1 M PIPES (pH 6.5), 1 mg

mL<sup>-1</sup> BSA, 1mM CaCl<sub>2</sub>, 1 mM NaN<sub>3</sub>. Samples taken at different times were analyzed by HPLC, first according to size on a TSK Gel Amide 80 column eluted with a 1:1 mixture of solution A (acetonitrile, H<sub>2</sub>O, 500 mM acetic acid triethylamine pH 7.3, 75/15/10, v/v/v) and B (acetonitrile, H<sub>2</sub>O, 500 mM acetic acid triethylamine pH 7.3, 50/40/10, v/v/v). The fractions were collected, dried and oligosaccharide isomers were fractionated on a Micropak-SP column eluted with solution C (100 mM acetic acid, 0.025% N-butanol, triethylamine, H<sub>2</sub>O, pH 4.0).

10 **Isolation and characterization of a novel human  $\alpha$ 1,2-mannosidase cDNA**

A novel human  $\alpha$ 1,2-mannosidase was identified by searching the EST database with the yeast  $\alpha$ 1,2-mannosidase amino acid sequence (Camirand, A. et al. (1991) *J. Biol. Chem.*, **266**, 15120-15127). The consensus sequence of the identified overlapping clones encodes the terminal 50 amino acids of the catalytic domain including two of the most highly conserved Class I  $\alpha$ -mannosidase sequence motifs followed by 589 bp of 3' UTR terminating in a poly A tail. Clones encoding the complete ORF (2.1 kb) and 5' UTR were then isolated by nested 5' RACE from human placenta, liver and testis cDNAs, as described in materials and methods. In addition an independent 2.7 kb clone containing the ORF flanked by 50 bp of 5' UTR and 589 bp of 3' UTR terminating with a polyA tail was isolated from a human fetal brain cDNA library. The sequence of the cDNAs obtained from the different sources were identical.

25 The 2.7 kb cDNA is predicted to encode a 79.5 kDa type II membrane protein with an 85 amino acid cytoplasmic tail, followed by a putative transmembrane domain of about 17 residues, a "stem" region of about 137 amino acids not required for enzyme activity and a large C-terminal catalytic domain (amino acids 240-699) (Fig. 1). The 5' UTR and ORF sequence were obtained from human placenta, testis and liver cDNA clones amplified by RACE, and a fetal brain cDNA library clone. The 3' UTR sequence is the consensus of an alignment of EST clones. Bold numbers refer to the deduced amino acid sequence and the numbers in

normal type refer to the nucleotide sequence. The conserved Class 1  $\alpha$ -mannosidase sequence motifs are underlined, and the highly conserved invariant acidic amino acid residues as well as the conserved cysteines common to all class I  $\alpha$ -mannosidases are boxed in grey. The putative transmembrane domain sequence is indicated in bold and underlined. Arrows indicate the starting amino acid residues of the different forms of the recombinant enzyme expressed in *Pichia pastoris*.

The cytoplasmic tail is much longer than any of the type II membrane-bound glycosidases or glycosyltransferases described so far, and contains a proline rich domain. The catalytic domain of this novel  $\alpha$ 1,2-mannosidase contains the highly conserved sequence motifs characteristic of Class I  $\alpha$ -mannosidases as well as the highly conserved disulfide bonded cysteines and acidic amino acid residues that are essential for enzymatic activity. The catalytic domain of the human  $\alpha$ 1,2-mannosidase is 43% identical (54% similar) to the yeast  $\alpha$ 1,2-mannosidase (Camirand, A. et al. (1991) *J. Biol. Chem.*, **266**, 15120-15127), and 40% identical (54% similar) to human and murine  $\alpha$ 1,2-mannosidase IA and IB. There is no significant similarity between the N-terminal sequence of this novel  $\alpha$ 1,2-mannosidase and previously cloned members of the same family.

Northern blot analysis indicates that all tissues examined expressed variable levels of a 3 kb transcript (Fig. 2). Random labeled  $\alpha$ 1,2-mannosidase EST clone R16652 was hybridized to Northern blots containing 2  $\mu$ g of poly (A<sup>+</sup>) RNA from human tissue. The blots were exposed to film to x-ray film for 5 days.

The expression is particularly high in testis and relatively low in lung and muscle. The gene encoding this novel  $\alpha$ 1,2-mannosidase is localized on chromosome 9 since a clone (T12605) isolated from a human chromosome 9 cosmid library contains exonic sequence identical to the cDNA sequence (nucleotides 731-916).

### Expression of recombinant $\alpha$ 1,2-mannosidase in *Pichia pastoris*

The  $\alpha$ 1,2-mannosidase was expressed as a secreted protein in *P.pastoris* in order to characterize its enzymatic activity. The catalytic domain starting at either amino acid 169 or 240 was cloned in frame downstream from the  $\alpha$ -factor of the *P.pastoris* expression vector pPICZ $\alpha$  A. Following methanol induction of yeast transformed with expression constructs pZ $\alpha$ ASHM169, pZ $\alpha$ ASHM240 or pZ $\alpha$ ASHM240T similar levels of  $\alpha$ 1,2-mannosidase activity were detected in the medium, and absent in yeast transformed with the pPICZ $\alpha$  A vector. Recombinant  $\alpha$ 1,2-mannosidase of the expected size (55 kDa) was observed by Western blot analysis at 2-3 days post-induction and decreased thereafter (Fig. 3). Ten microlitres of medium was subjected to reducing SDS-PAGE (10%) and the recombinant Myc tagged  $\alpha$ 1,2-mannosidase was visualized by Western blot analysis. Lanes 1-4 correspond to GS115 transformed with pZ $\alpha$ ASHM240T at 2, 3, 4, and 5 days post-induction, respectively. Lane 5 corresponds to GS115 transformed with vector at 5 days post-induction. Molecular mass standards are indicated on the right.

### Properties of the human $\alpha$ 1,2-mannosidase

The catalytic properties of the recombinant  $\alpha$ 1,2-mannosidase were studied using [ $^3$ H]Man $_9$ GlcNAc as substrate. The enzyme is active over a pH range of 6.3-7.2 with an optimum between 6.5-6.9. Inclusion of at least 0.1 mM Ca $^{2+}$  in the assay is required to obtain maximum enzyme activity. The enzyme is inhibited 50% by 1  $\mu$ M EDTA and 100% by 25  $\mu$ M EDTA. This inhibition is completely reversed by the addition of 2 mM Ca $^{2+}$  but, not by 2 mM Mn $^{2+}$ , Mg $^{2+}$ , Zn $^{2+}$  or Co $^{2+}$ . The  $K_m$  of the recombinant enzyme is 0.4 mM which is similar that of the recombinant yeast  $\alpha$ 1,2-mannosidase (0.3 mM).

The human  $\alpha$ 1,2-mannosidase is inhibited by the Class I  $\alpha$ -mannosidase inhibitors, 1-deoxymannojirimycin (IC $_{50}$  = 75  $\mu$ M) and



kifunensine ( $IC_{50} = 70$  nM), but is insensitive to the Class II  $\alpha$ -mannosidase inhibitor swainsonine (Table I).

**Table I**

Effect of  $\alpha$ -mannosidase inhibitors on the human  $\alpha$ 1,2-mannosidase activity

Inhibitor	Activity <sup>a</sup> (% of control)
1-deoxymannojirimycin	
10 $\mu$ M	84
50 $\mu$ M	62
500 $\mu$ M	10
kifunensine	
0.05 $\mu$ M	67
0.10 $\mu$ M	28
0.50 $\mu$ M	0
swainsonine	
5 $\mu$ M	100
10 $\mu$ M	100
100 $\mu$ M	100

<sup>a</sup> Assay conditions are described in materials and methods. Activity is expressed as a percentage of [<sup>3</sup>H]mannose released in the absence of inhibitors (226 dpm).

### Specificity of the human $\alpha$ 1,2-mannosidase

The recombinant  $\alpha$ 1,2-mannosidase was incubated with [<sup>3</sup>H]Man<sub>9</sub>GlcNAc for different periods of time and HPLC analysis showed that the only products formed are Man<sub>8</sub>GlcNAc and mannose in a time dependent manner (Fig. 4). [<sup>3</sup>H]Man<sub>9</sub>GlcNAc was incubated with medium obtained from *P.pastoris* transformed with pZ $\alpha$ ASHM169 two days post-induction. Oligosaccharide product formation was monitored by HPLC as described in materials and methods and is expressed as a percentage of the total radioactivity recovered.

Supplementing the incubation mixture with fresh enzyme after 8 and 22.5 hours of incubation did not result in any further mannose trimming. The Man<sub>8</sub>GlcNAc oligosaccharide formed was demonstrated to be isomer B by [<sup>1</sup>H]-NMR analysis (Fig. 5). Spectrum at 600 MHz and 30°C

of the anomeric region of the  $\text{Man}_8\text{GlcNAc}$  produced by the hydrolysis of  $\text{Man}_9\text{GlcNAc}$  by the specific human  $\alpha 1,2$ -mannosidase. The resonance corresponding to the anomeric proton of each mannose residue is numbered. Integral values for each of the anomeric protons, except  
5  $\text{GlcNAc}$ , were obtained. The split resonance signal at 5.104 and 5.075 ppm for residue 7 is characteristic of  $\text{Man}_8\text{GlcNAc}$  isomer B.

Consistent with the strict specificity of this enzyme,  $\text{Man}_8\text{GlcNAc}$  isomer B and p-nitrophenyl- $\alpha$ -D-mannopyranoside are not substrates of the enzyme. Therefore this highly specific  $\alpha 1,2$ -mannosidase is the human  
10 ortholog of the yeast processing  $\alpha 1,2$ -mannosidase that has been implicated in the targeting of misfolded glycoproteins for degradation (Knop, M. et al. (1996) *Yeast*, **12**, 1229-1238; Jakob, C.A. et al. (1998) *J. Cell Biol.*, **142**, 1223-33). Since there is evidence that *N*-glycan trimming by 1-deoxymannojirimycin and kifunensine-sensitive ER  $\alpha 1,2$ -mannosidase  
15 activity is also implicated in the degradation of misfolded glycoproteins in mammalian cells (Su, K et al. (1993) *J. Biol. Chem.*, **268**, 14301-14309; Liu, Y. et al. (1997) *J. Biol. Chem.*, **272**, 7946-7951; Yang, M. et al. (1998) *J. Exp. Med.*, **187**, 835-846; Liu, Y. et al. (1999) *J. Biol. Chem.*, **274**, 5861-5867), it seems likely that the novel human  $\alpha 1,2$ -mannosidase we have  
20 cloned is involved in ER quality control, but this remains to be shown.

### Characterization of the human R461L $\alpha 1,2$ -mannosidase mutant

The medium expressing the catalytic domain of the R461L mutant was incubated with  $\text{Man}_9\text{GlcNAc}_2$ -PA for different times and the  
25 products were fractionated by HPLC with fluorescence monitoring (due to the PA fluorescent tag). The R461L mutant removes at least three mannose residues from  $\text{Man}_9\text{GlcNAc}_2$  (Fig. 6), unlike the parent ER  $\alpha 1,2$ -mannosidase that forms  $\text{Man}_9\text{GlcNAc}_2$  (Fig. 4). The products of the reaction are shown fractionated by HPLC (Fig. 6). Analysis of the  
30 oligosaccharide isomers by HPLC indicates the pathway of mannose

removal shown in Fig. 7. In recent work, we had demonstrated that the corresponding mutation in the yeast endoplasmic reticulum  $\alpha$ 1,2-mannosidase (yeast R273L mutant) also changes the specificity of the yeast enzyme (Romero, P.A. et al. (2000) *J. Biol. Chem.* **275**: 11071-11074).

The present invention will be more readily understood by referring to the following examples which are given to illustrate the invention rather than to limit its scope.

10

### EXAMPLE I

Possible approaches whereby cloning and expression of this new human  $\alpha$ 1,2-mannosidase can be used to design new specific inhibitors that might be developed for drug therapy of glycoprotein folding diseases:

- 15 (1) use of recombinant enzyme for high throughput screening of naturally occurring compounds as potential specific inhibitors.
- (2) use of recombinant enzyme to test chemically synthesized derivatives of mannodeoxynojirimycin and kifunensine as potential specific inhibitors.
- (3) molecular modelling of the human mannosidase based on its amino  
20 acid sequence and the recently determined three-dimensional structure and identification of the active site of the yeast  $\alpha$ 1,2-mannosidase ortholog that has the same properties, specificity and function in degradation of misfolded glycoproteins.
- (4) Expression of mutant ER  $\alpha$ 1,2-mannosidase can alter carbohydrate  
25 structure of recombinant mammalian glycoproteins expressed for therapeutic purposes to improve their uptake by mammalian cells.

While the invention has been described in connection with specific embodiments thereof, it will be understood that it is capable of further modifications and this application is intended to cover any variations, uses, or adaptations of the invention following, in general, the  
30

principles of the invention and including such departures from the present disclosure as come within known or customary practice within the art to which the invention pertains and as may be applied to the essential features hereinbefore set forth, and as follows in the scope of the

5 appended claims.

**WHAT IS CLAIMED IS:**

1. A human  $\alpha$ 1,2-mannosidase enzyme for specifically converting  $\text{Man}_9\text{GlcNAc}$  to  $\text{Man}_8\text{GlcNAc}$  isomer B in degradation mechanism of misfolded proteins, wherein said enzyme has the characteristics of an enzyme encoded by a cDNA sequence set forth in Fig. 1.
2. An agonist or antagonist of the  $\alpha$ 1,2-mannosidase enzyme of claim 1 for activating or inhibiting said enzyme.
3. The agonist or antagonist of claim 2, wherein said activating or inhibiting is for a transient period of time.
4. An antagonist of claim 2, wherein said inhibiting is for a transient period of time, thereby preventing degradation of misfolded glycoproteins.
5. A method for the treatment of a genetic disease causing a misfolding of proteins in a patient, which comprises administering an antagonist of  $\alpha$ 1,2-mannosidase enzyme of claim 1 for transiently inhibiting said enzyme, thereby preventing degradation of misfolded glycoproteins.
6. The method of claim 5, wherein said genetic disease is selected from the group consisting of cystic fibrosis and emphysema.
7. The method of claim 6, wherein for cystic fibrosis said misfolded protein is cystic fibrosis transmembrane conductance regulator (CFTR).
8. The method of claim 6, wherein for emphysema said misfolded protein is alpha1 antitrypsin.
9. The use of a mutant  $\alpha$ 1,2-mannosidase to produce altered recombinant glycoproteins with improved uptake.

10. The use of claim 9, wherein said improved uptake is to treat genetic diseases characterized by formation of misfolded glycoproteins resulting in their degradation and/or improper localization.

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List

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2/9

TTCAGTGAGTGGTTGGCCTCGGTCTCAGACTGATCGAGCGGCTGGACACCATGTGGATCTTGGTCTGAGGAAGAATTTGAGGAGCCAGGAAGTGGGTGTGGAAGATTACACTTT  
960  
F S E W F G L G L T L I D A L D T M W I L G L R K E F E A R K W V S K K L H F 320

GAAAAGCAGCTGAGCTCAACCTGTTGAGAGCAGCATCCGATCCTGGGGGGCTCCTGAGTGCTACCACTGTCTGGGACAGCTCTTCTGAGGAAGCTGAGGATTTTGGAAAT  
1080  
E K D V D V N L F E S T I R I L G G L L S A Y H L S G D S L F L R K A E D F G N 360

CGCTAATGCCCTTCAGAACACCATCCAGATTCTTACTCGGATGTGAACATCGGTACTGGAGTTGCCACCGCCACGGTGGACCTCCGACACACTGTGGCCGAGGTGACCAGC  
1200  
R L M P A F R T P S K I P Y S D V N I G T G V A H P P R W T S D S T V A E V T S 400

ATTCAGTGGAGTCCGGAGCTCTCCCTCTCAGAGGGGATAACAAGTTTCAGGAGGCTGGAGAAGGTGACACAGCACATCCAGGCCCTGTCTGGGAAGAGGATGGGTGGTGGCC  
1320  
I Q L E F R E L S R L T G D K K F Q E A V E K V T Q H I H G L S G K K D G L V P 440

ATGTTTCATACCCACAGTGGCTCTTACCCACCTGGCGGTATTCACGCTGGCGGCCAGGCCACAGCTACTATGAGTACCTGCTGAAGCAGTGGATCCAGGGCGGGAGCAGGAG  
1440  
M F I N T H S G L F T H L G V F T L G A R A D S Y Y E Y L L K Q W I Q G G K Q E 480

ACACAGCTGCTGGAAGCTACGTGGAAGCCATCGAGGGTGTGAGAGCCACCTGCTGGGGCACTCCGAGCCCACTAAGCTCACCTTTGTGGGGAGCTTGGCCACGGCCGCTTCAGTGGC  
1560  
T Q L L E D Y V E A I E G V R T H L L R H S E P S K L T F V G E L A H G R F S A 520

AAGATGGACCACTGCTGCTCCAGGGAGCGTGGCTCTGGCGGTCTACAGGGCTGCGCCGACATGAGCTGGCCAGGAGCTCATGAGACTTGTACAGATGAAC  
1680  
K M D H L V C F L P G T L A L G V Y H G L P A S H M E L A Q E L M E T C Y Q M N 560

FIG. 1 (cont.)

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CGGAGATGAGACGGGGCTGAGTCCCGAGATCGTGCACCTTCAACCTTACCCCAAGCCGGGCGCTGGAGGTCAAGCCAGCAGAGGACACACCTGCTGGGGCCAGAGACC 1800  
R Q M E T G L S P E I V H F N L Y P Q P G R R D V E V K P A D R H N L L R P E T 600  
GTGGAGAGCCTGTTCTACCTGTACCGGCTACAGGGGACCGCAATACACGAGCTGGGGCTGGGAGATTCTGCAGAGCTTCAGCCGATTACACAGGGTCCCTCGGGTGGCTATTCTTCC 1920  
V E S L F Y L Y R V T G D R K Y Q D W G W E I L Q S F S R F T R V P S G G Y S S 640  
ATCAACAATGTCAGGATCCTCAGACGCCGAGCCTAGGACAAGATGGAGAGCTTCTTCTGGGGGAGACGCTCAAGTATCTGTCTTCTCTCGATGACCCCAACCTGCTCAGC 2040  
I N N V Q D P Q K P E P R D K M E S F F L G E T L K Y L F L L F S D D P N L L S 680  
CTGGAGCCTAGCTGTTCAACACCGAAGCCCAACCTCTGCCTATCTGACCCCTGCTAGGTGGATGGCTGCTGGTGGGAGCTTCGGGTGGGCAGAGGCACCTTGCCTGGGTCTGTGG 2160  
L D A Y V F N T E A H P L P I W T P A \* 699  
CATTTTCAAGGCCACGTAGCACCGGCAACCGCCAGTGGCCACAGGCTCTGAATGGCTCTGGGCTCTCTGCTCTCTGCTTTAATCAGGACACCGTGAGGACAAGTGAGGCCGTGAG 2280  
TCTTGGTGTGATGCGGGGTGGGCTGGCCGCTGGAGCCTCCGCTGCTTCTCCAGAGACACCAATCATGACTACCATTCGCTGAGCCTGAGCAGGTCTCTGTGGGCCGACAGAGGG 2400  
GGGCTTCGAGGTGCTCCCTGTTACTGGGGTGACCGAGTGGACAGCCAGGGTGCAGCTCTGCCCGGGCTCGTGAAGCCTCAGRTGTCCCCAATCCAAGGCTCTGGAGGGGCTGCCGTGAC 2520  
TCCAGAGGCCCTGAGGCTCCAGGGCTGGCTCTGGTGTTTACAGCTGGAATCAGGGATCCTCTGGCCGCCCGCCAGGGGCTTGGAGGGCTGGAGGCAAGTCCGCTCTAGCTCAGGGGCC 2640  
CCTCCAGTGAATGGGTCTTTTCGGTGGAGATAAAGTTGATTGCTCT 2689

~~FIGURE 1~~ (cont.)

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28 DEC 2009

leukocytes  
colon  
intestine  
ovary  
testis  
prostate  
thymus  
spleen  
pancreas  
kidney  
muscle  
liver  
lung  
placenta  
brain  
heart

kb

9.5 -

7.5 -

4.4 -

2.4 -

1.35 -

kb

9.5 -

7.5 -

4.4 -

2.4 -

1.35 -

Fig. 2

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28 DEC 2001

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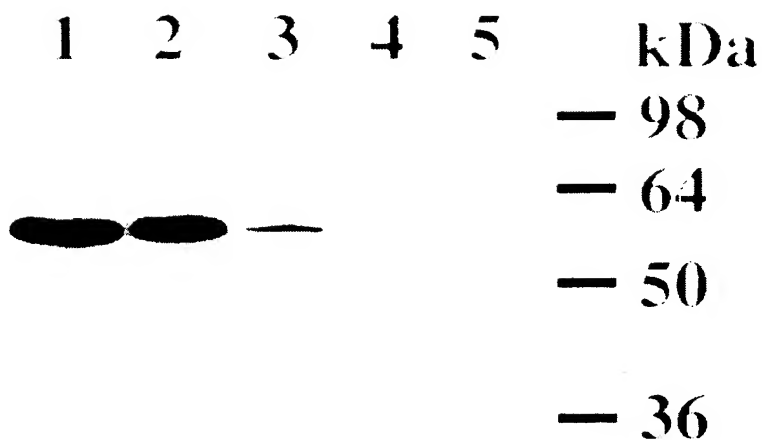
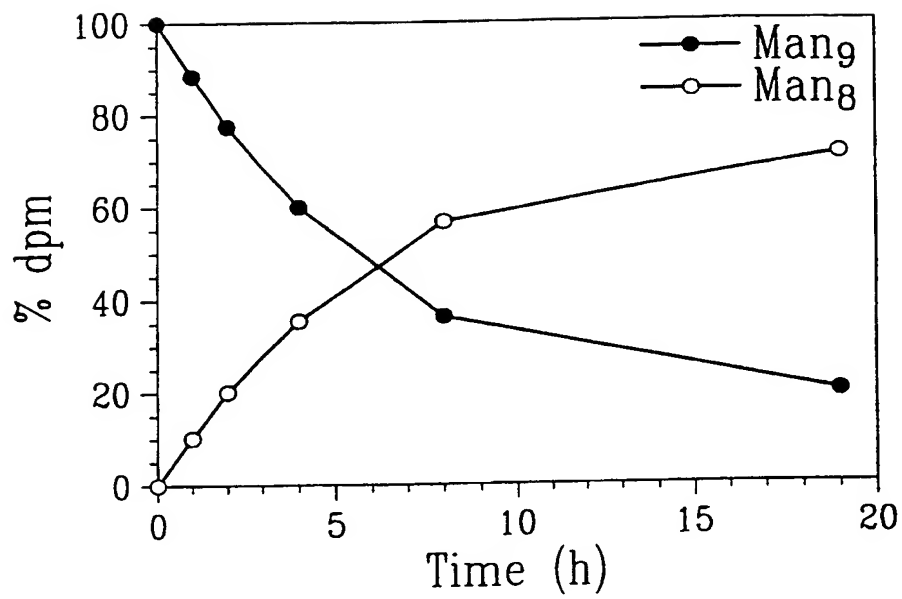


FIG. 3

531 Rec'd PCT/PTC 28 DEC 2004

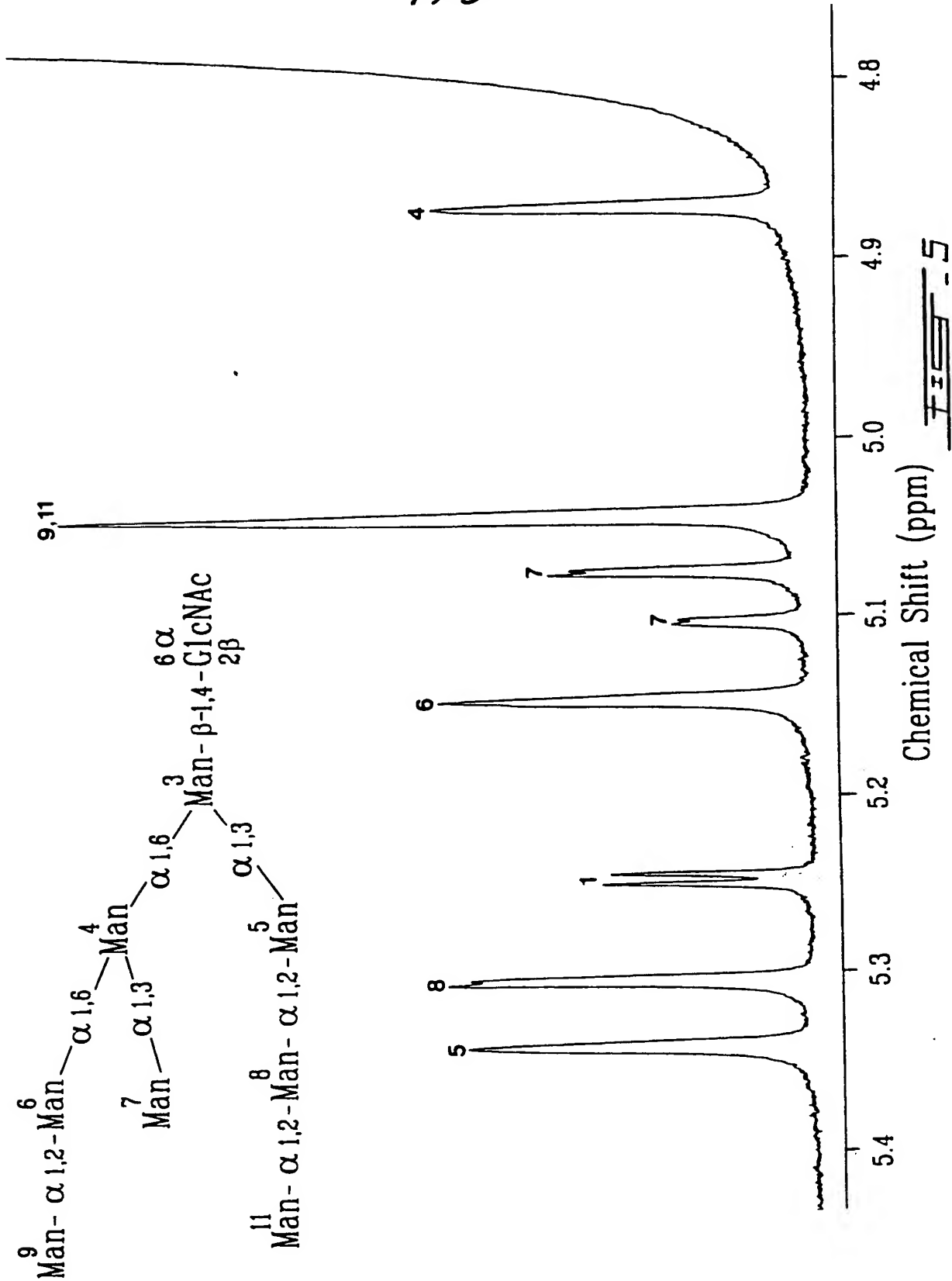


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FIG. 4

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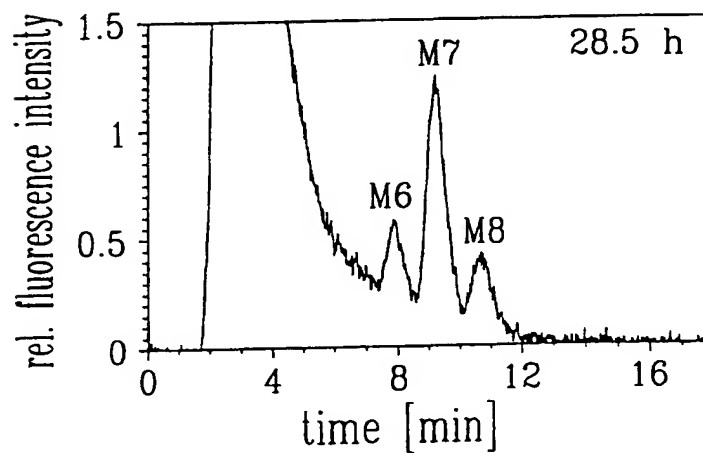
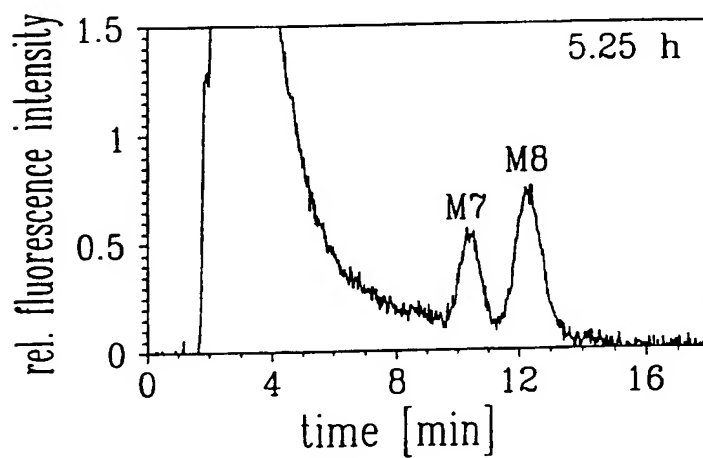
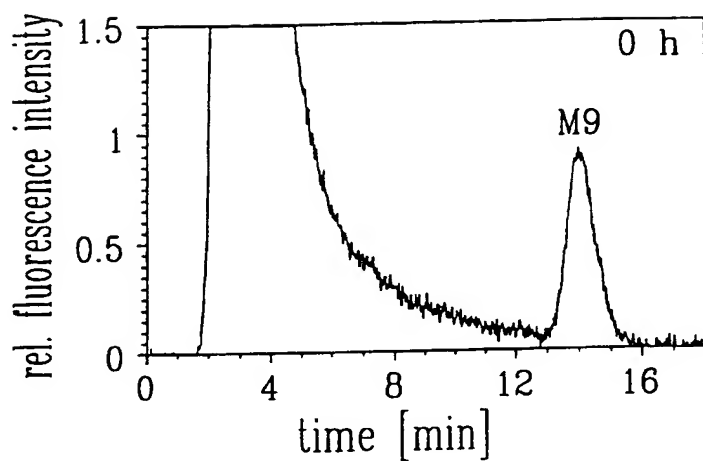


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28 DEC 2001

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R461L



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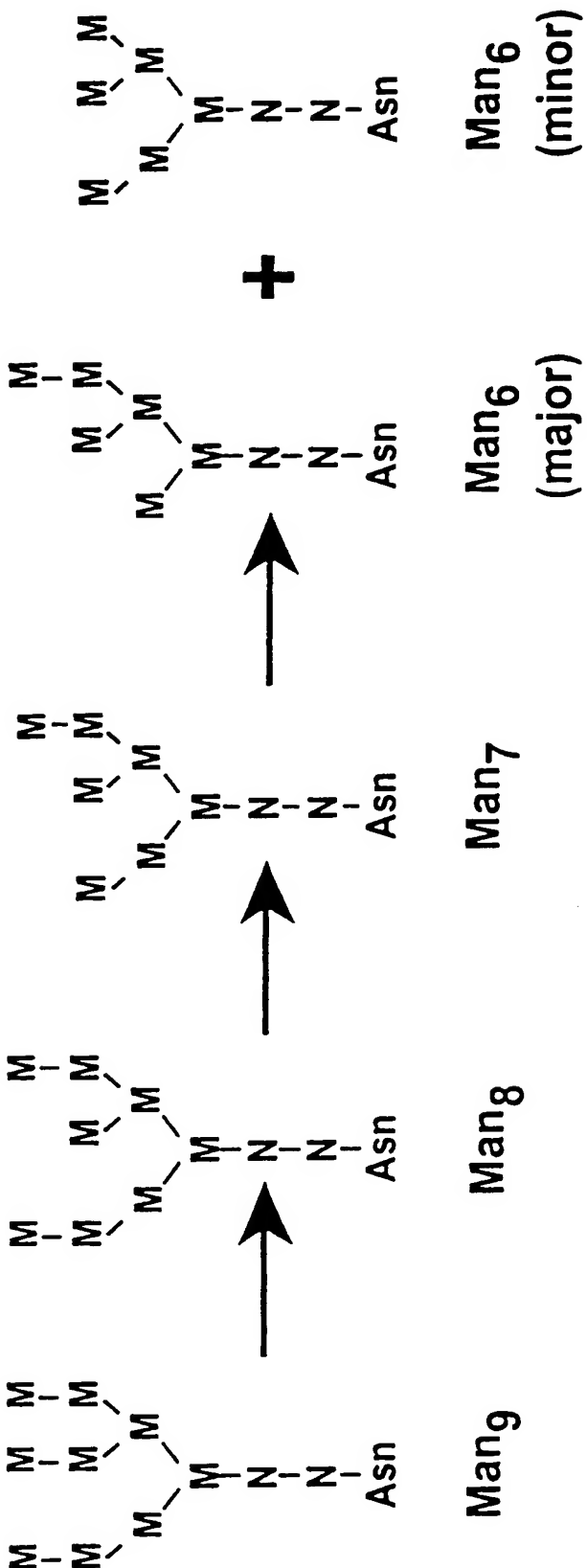


FIG. 7

531 Rec'd PCT/PTC 28 DEC 2001



# INTERNATIONAL SEARCH REPORT

International Application No  
PCT/CA 00/00775

## A. CLASSIFICATION OF SUBJECT MATTER

IPC 7 C12N15/56 C12N9/24 C12P21/00

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 7 C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

BIOSIS, EPO-Internal, EMBL, STRAND, WPI Data, PAJ, CHEM ABS Data, MEDLINE

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	<p>LAL ANITA ET AL: "Substrate specificities of recombinant murine Golgi alpha1,2-mannosidases IA and IB and comparison with endoplasmic reticulum and Golgi processing alpha1,2-mannosidases." GLYCOBIOLOGY, vol. 8, no. 10, October 1998 (1998-10), pages 981-995, XP000952920 ISSN: 0959-6658 abstract page 986, left-hand column, last paragraph -right-hand column, paragraph 1 page 989, right-hand column figure 11</p> <p style="text-align: center;">---</p> <p style="text-align: center;">-/--</p>	1

☒ Further documents are listed in the continuation of box C.

☒ Patent family members are listed in annex.

\* Special categories of cited documents :

- "A" document defining the general state of the art which is not considered to be of particular relevance
- "E" earlier document but published on or after the international filing date
- "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- "O" document referring to an oral disclosure, use, exhibition or other means
- "P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search

20 October 2000

Date of mailing of the international search report

27. 10. 00

Name and mailing address of the ISA

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Authorized officer

Lejeune, R

# INTERNATIONAL SEARCH REPORT

International Application No

PCT/CA 00/00775

## C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	<p>WENG SHUAI ET AL: "Evaluation of the early processing routes of N-linked oligosaccharides of glycoproteins through the characterization of Man-8GlcNAc-2 isomers: Evidence that endomannosidase functions in vivo in the absence of a glucosidase blockade."            GLYCOBIOLOGY,            vol. 6, no. 8, 1996, pages 861-868,            XP000952947            ISSN: 0959-6658            abstract            page 865, right-hand column, paragraph 2            page 864, left-hand column, last line            -right-hand column, paragraph 1            figure 6</p>	2-4
A	<p>---            DATABASE EMBL [Online]            Accession AA631254,            31 October 1997 (1997-10-31)            NCI-CGAP: "nq81c12.s1 NCI CGAP Co9 Homo sapiens cDNA clone IMAGE:1158742 3' similar to WP:T03G11.4 CE04872 MAN(9)-ALPHA-MANNOSIDASE ;, mRNA sequence."            XP002150707            cited in the application            96.7% identity in 880 BP overlap with SEQ ID NO 18</p>	1
A	<p>---            JAKOB CLAUDE A ET AL: "Degradation of misfolded endoplasmic reticulum glycoproteins in Saccharomyces cerevisiae is determined by a specific oligosaccharide structure."            JOURNAL OF CELL BIOLOGY,            vol. 142, no. 5, pages 1223-1233,            XP002150705            ISSN: 0021-9525            abstract</p>	5-10
P,X	<p>---            TREMBLAY LINDA O ET AL: "Cloning and expression of a specific human alpha1,2-mannosidase that trims Man9GlcNAc2 to Man8GlcNAc2 isomer B during N-glycan biosynthesis."            GLYCOBIOLOGY,            vol. 9, no. 10, October 1999 (1999-10),            pages 1073-1078, XP000952919            ISSN: 0959-6658            the whole document</p> <p style="text-align: center;">--- -/--</p>	1-4

# INTERNATIONAL SEARCH REPORT

International Application No

PCT/CA 00/00775

## C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P,X	<p>GONZALEZ DANIEL S ET AL: "Identification, expression, and characterization of a cDNA encoding human endoplasmic reticulum mannosidase I, the enzyme that catalyzes the first mannose trimming step in mammalian Asn-linked oligosaccharide biosynthesis."  JOURNAL OF BIOLOGICAL CHEMISTRY,  vol. 274, no. 30,  23 July 1999 (1999-07-23), pages  21375-21386, XP002150706  ISSN: 0021-9258  the whole document</p>	1-4
P,X	<p>WO 00 12708 A (BAKER KEVIN ; GENENTECH INC (US); GODDARD AUDREY (US); GURNEY AUSTI)  9 March 2000 (2000-03-09)  page 3  page 46 -page 48  figures 9,10; example 8  100% identity between the AA sequence of PR01477 and SEQ ID 19.  99.9% identity between the nucleic acid sequence of PR01477 and SEQ ID 18</p>	1
E	<p>WO 00 58473 A (CURAGEN CORP ; LEACH MARTIN (US); SHIMKETS RICHARD A (US))  5 October 2000 (2000-10-05)  page 231  99.9% identity in 2712 BP overlap between SEQ ID 3585 of W00058473 and SEQ ID NO 18  100% identity in 663 AA overlap between SEQ ID 3586 of W00058473 and SEQ ID NO 19</p>	1

## INTERNATIONAL SEARCH REPORT

International application No.  
PCT/CA 00/00775

### Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☒ Claims Nos.:  
because they relate to subject matter not required to be searched by this Authority, namely:  
Although claims 5-8 are directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.
2. ☐ Claims Nos.:  
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:
3. ☐ Claims Nos.:  
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

### Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

#### Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.
- ☐ No protest accompanied the payment of additional search fees.

# INTERNATIONAL SEARCH REPORT

information on patent family members

International Application No

PCT/CA 00/00775

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 0012708 A	09-03-2000	AU 5590899 A	21-03-2000
		AU 6041399 A	10-04-2000
		WO 0017353 A	30-03-2000
-----			
WO 0058473 A	05-10-2000	NONE	
-----			

